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THE INFLUENCE OF INDUCED HYPERTHYROIDISM ON EXPERIMENTAL TUBERCULOSIS IN MICE

BY

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To the Memory of my Father

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Alf Backman

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INTRODUCTION

During the period of a hundred years or so in which problems relating to resistance and susceptibility to infections have been subjected to study, the role of the endocrine system in these phenomena has attracted considerable interest.

When the aim of the investigation has been to study the relations of the organism to incipient and progressive infection, the choice has tended to fall on tuberculous infection, owing to its usually chronic character.

Among other endocrine factors, the function of the thyroid gland in connection with a tuberculous infection has attracted attention. This interest originated in certain observations made by experienced clinicians, who found that slight hyperthyroidism in connection with incipient tuberculous infection was a favourable sign from the standpoint of prognosis, and that a goitre in a patient with tuberculosis should be left intact. Thus, the conclusion was drawn that a relationship existed between thyroid function and tuberculous infection.

The large number of clinical, patho-anatomical and endocrinological observations reviewed by Schäfer (110) and in part by Rich (105) and Lurie (66) strongly suggest that thyroid function does play a part in the response of the body to tuberculous infection. In association with incipient tuberculous infection, a reactive hyperthyroidism often forms an element of the general sympathicotonic phase of defence and subsides during the subsequent course of the disease. The significance of this reactive hyperthyroidism from the standpoint of prognosis has aroused considerable interest, and the general belief is that it indicates effective resistance on the part of the organism to the tuberculous infection. It should be mentioned, however, that dissenting views have been expressed.

Numerous attempts have been made to elucidate this problem by means of animal experiments, but the results so far obtained are conflicting.

SURVEY OF THE LITERATURE

Thyroid function and experimental tuberculosis

Since the clinical data relating to the influence of thyroid function on tuberculous infection are contradictory, experimental investigations have been undertaken to throw light on this question. Hyperthyroidism and hypothyroidism have been induced in animals in various ways, and the course of a tuberculous infection has then been studied. The advantage of these animal experiments is that the dose used for inoculation can be quantitatively determined and the dosage varied.

Hyperthyroidism

Hyperthyroidism has been induced by the administration of thyroid (pulverized dried thyroid gland substance) or purified preparations of thyroid hormone. By noting gross changes and survival time in experiments with rabbits, Frugoni & Grixoni (30), Schröder (114) and Dahl (13) arrived at the conclusion that hyperthyroidism has a mitigating effect on tuberculous infection. Similar results were obtained by Lurie, Zapposodi & Levy (72) and Lurie & Ninos (67), who utilized histological criteria in addition to those mentioned above, *i.e.* gross lesions and survival time.

In experiments with guinea-pigs an ameliorating effect of hyperthyroidism on a tuberculous infection was noted by Webb, Gilbert & Ryder (143), Izzo & Cicardo (39) and Gädeke & Jakob (31). Schäfer (109) and Wasz-Höckert, Backman & Poppius (136) reported that slight hyperthyroidism induced with small doses of thyroid reduced the severity of a tuberculous infection, whilst larger doses, causing more marked hyperthyroidism, led to exacerbation of the infection. In these studies, too, the ensuing gross organ lessions were used as a criterion.

In experiments with rats, Glyone (33) showed that intraperitoneally injected *M. tuberculosis* bacilli were rapidly eliminated from the abdominal cavity of animals treated with thyroid. By contrast, he could find no effect of thyroid medication on the resulting pulmonary lesions. Glyone also described experiments with the axolotl, which is completely resistant to tuberculosis. Under the influence of thyroid, *M. tuberculosis* bacilli were eliminated even more rapidly.

However, a definitely aggravating effect of hyperthyroidism on experimental tuberculosis has also been reported by a number of investigators. Evaluating survival time and gross lesions, Schedtler (111, 112), using rabbits as experimental animals, and Freud (29), Schedtler (111, 112) and Swedberg (126), using guinea-pigs, noted an obviously noxious influence of thyroid. Using the same criteria, Schäfer (109) and Wasz-Höckert *et al.* (136) observed this deleterious effect only in marked experimental hyperthyroidism induced with large doses of thyroid. In experiments with mice, Dubos (17) and Nutter, Gemmill & Myrvik (90) found that hyperthyroidism reduced the survival time in tuberculosis. When, in addition to the survival time, the microbial content of the organs was quantitatively determined in experiments with mice, a definite aggravation of the tuberculous infection was noted in animals with induced hyperthyroidism [Smith & Dubos (123)].

Hypothyroidism

The effect of hypothyroidism has also attracted a great deal of interest. This state has been induced by thyroidectomy or by inhibiting the synthesis of the thyroid hormone with thyrostatic drugs, in particular methyl- and propylthiouracil.

An improvement after thyroidectomy in rabbits and guinea-pigs with experimental tuberculosis was reported by Schedtler (111, 112), whilst Izzo & Cicardo (39), in experiments with guinea-pigs, failed to note any effect of thyroidectomy.

A definitely exacerbating effect of thyroidectomy on an experimental tuberculous infection was observed by Schröder (114) and Lurie, Zapposodi & Blaker (69) in rabbits, by Webb, Gilbert & Ryder (143) and Kallós & Kentzler (41) in guinea-pigs and by Steinbach (125) in rats.

Hypothyroidism induced with thyrostatic drugs (methylthiouracil, propylthiouracil and thiourea preparations) has attracted interest

especially because these drugs and their derivatives are known to have a bacteriostatic effect *in vitro*. In experiments with mice Mayer, Eisman & Kanopka (86) and Eisman, Kanopka & Mayer (25) noted a good effect of substituted thiourea and thiouracil preparations in experimental tuberculosis.

Aggravation of the tuberculous infection in rabbits treated with propylthiouracil was reported by Lurie, Zapposodi & Blaker (69). In experiments with guinea-pigs Schäfer (109), Gädeke & Jakob (31) and Wasz-Höckert *et al.* (136) found that methylthiouracil had a deleterious influence in experimental tuberculosis.

The mode of action of induced hyperthyroidism and hypothyroidism in experimental tuberculosis

The question of how experimental hyper- and hypothyroidism influence tuberculous infection has been much discussed. As a link in the chain of studies concerning these problems, investigations relating to the effect of the states on tuberculin-allergic sensitivity have been carried out. Kallós & Kentzler (41) and Taubenhaus & Amronin (128) reported a definite increase in tuberculin sensitivity in animals which had received thyroid. Similar results were obtained by Long & Miles (63) and Long, Miles & Perry (64), who found, in addition, that ACTH and cortisone have an antagonistic effect. A decreased tuberculin allergic sensitivity in experimental hypothyroidism induced with propylthiouracil was noted by Lurie & Ninos (67) and Lurie, Zapposodi & Blaker (69). After thyroidectomy the inhibiting effect of hypothyroidism on the tuberculin-allergic sensitivity was still more marked than after thiouracil medication [Lurie, Zapposodi & Blaker (69)].

An enhancement of the anti-inflammatory reaction in the tissues due to thyroid has been noted in connection with tuberculous infection by Schmid (113), Lurie (66), Masalinski (85) and Lurie, Zapposodi & Levy (72), in studies with diphtherial toxin by Long & Schevell (65) and with the use of India ink by Lurie & Ninos (67). Conversely, too, this relationship between thyroid function and anti-inflammatory reactions has been demonstrated, increased thyroid activity having been noted by Eichof (24) after immunization of guinea-pigs with heterologous sera. That thyroid stimulates the phagocytosis of *M. tuberculosis* bacilli has been unequivocally demonstrated by Glyone (33) and Lurie & Ninos (67).

An effect of thyroid implying that the tissues are influenced in such a way that a rapid spread of the bacilli occurs and that concomitantly the formation of gross lesions is prevented was reported by Steinbach (125) and Lurie, Zapposodi & Levy (72) in animal experiments. But although a »transport of microbes towards the lymph nodes» was seen, no decrease in the number of bacilli per unit of tissue was noted. By contrast, Taubenhaus & Amronin (128) emphasized that thyroid obviously stimulates the formation of granulation tissue, and thus probably the encapsulation of the tuberculous foci. According to Masalinski (85), an increase in the number of fibroblasts in the tissues is also seen in experimental tuberculosis in association with thyroid medication.

In studies concerning the enzymatic system of the *M. tuberculosis* bacillus and related factors, it has been found that lysozyme has a lethal effect on this microbe and that the lysozyme content increases following a BCG vaccination [Myrvik & Weisen (89)]. On the other hand, it has been shown that thyroid inhibits lysozyme activity [Litwack (62)]. From experiments with mice revealing a deleterious effect of thyroid in tuberculosis, Nutter, Gemmill & Myrvik (90) concluded that this result was due to inhibition of lysozyme activity by this hormone.

Pätiälä (92) and Kerppola & Pätiälä (46) reported that the concentrations of coenzyme I and coenzyme II decreased in the blood of guinea-pigs with experimental tuberculosis. In man, too, in a series of patients with tuberculosis, decreased values for the concentrations of these enzymes in the blood were noted.

Studies on the mode of action of thiouracil and thiourea preparations revealed that thiouracil diminishes tuberculin sensitivity and general anti-inflammatory activity and suppresses phagocytic activity [Lurie & Ninos (67)]. An effect of thiouracil on tuberculous infection resembling that of cortisone has been reported by certain investigators [Lurie & Ninos (67), Lurie, Zapposodi & Blaker (69)]. On the other hand, an inhibitory effect on the oxidative enzymes of the *M. tuberculosis* bacillus, cytochrome C and succino-oxidase, has been observed after thiouracil treatment [Andrejew (4)].

By purely bacteriological methods it has been shown that both thiourea and thiouracil have a tuberculostatic effect *in vitro* [Liebmeister (69)]. A tuberculostatic effect *in vitro* has also been produced with thyroxine-like chemical substances, but not with thyroxine or thyroid extract [Barry (7)].

Attempts have been made to elucidate the endocrinological mechanism by which hyperthyroidism and hypothyroidism influence infection by investigating the stress syndrome and the endocrinological interrelations under the influence of thyroid and thiouracil.

According to Selye (116, 117), thyroid function is decreased in stress, and similar results have been reported by other writers [Paschkis *et al.* (97), Harris (35)]. In experiments with rats, Reichlin & Glaser (104) found that both streptococcal infection and traumatic stress lead to inactivation of the thyroid gland. By contrast, Uotila & Pekkarinen (129) showed that the thyroid is mobilized in an early stage of stress, and Gädeke & Jakob (31) reported that this is also the case in tuberculous infection.

In histoquantitative investigations of the thyroid gland in guinea-pigs with experimental tuberculosis, Pätiälä & Isotalo (93, 94) were able to show that thyroid function was inhibited. Similar results have been reported by Kracht & Spaethe (53) in rabbits with experimental tuberculosis.

In studies concerning thyroid function in guinea-pigs with experimental tuberculosis, Lehto (60) noted a slight increase in the uptake of radioactive phosphorus, P^{32}, by the thyroid gland during the generalized phase of the infection, when the values for this uptake were corrected by the blood values. This seems to indicate that a slight reactive hyperthyroidism occurs in incipient tuberculous infection.

Hypertrophy of the adrenal cortex due to the influence of thyroid has been observed by a number of investigators [Deane & Greep (14), Long & Miles (63), Kracht & Spaethe (53), Money (88)].

Atrophy or inactivation of the adrenal cortex caused by thiouracil has been reported by Leblond & Hoff (59), Deane & Greep (14), Zarrow & Money (155), Kracht & Spaethe (53) and Money (88).

The adrenal medulla is also influenced by thyroid. In mice with experimental hyperthyroidism its volume has been found to be unchanged, whilst a decrease has been noted in the concentration of both noradrenaline and adrenaline. On thiouracil treatment noradrenaline and adrenaline again increased [Hopsu (37)].

A definite aggravation of tuberculous infection in animals treated with ACTH or cortisone has been reported by a large number of writers, *e.g.* Bloch *et al.* (10), Lurie *et al.* (70, 71), Lurie & Zapposodi (68) and Batten & McCune (8). These investigators pointed out that since

interaction obviously occurs between the thyroid gland and the pituitary-adrenocortical system, ACTH and cortisone may well afford the clue to the influence of thyroid function on tuberculous infection.

Thyroid function and non-tuberculous infections

The influence of thyroid function has lately been studied in other infections besides tuberculosis.

A favourable effect of experimental hyperthyroidism induced by thyroid medication was seen in mice with poliomyelitis. The effect was good in young mice but uncertain in older animals [Holtman (36)]. In mice mildly infected with *Brucella melitensis* improvement was noted after treatment with trioidothyronine, whilst this treatment had a deleterious effect in heavy infection [Melby *et al.* (87)].

In mice, induced hyperthyroidism has been found to exacerbate staphylococcal infection [Dubos (16), Smith & Dubos (123)] streptococcal infection [Smith & Dubos (123)], pneumococcal infection [Smith & Dubos (123), Nutter, Gemmill & Myrvik (90)], dysentery and salmonellosis [Smith & Dubos (123)], poliomyelitis (Lancing virus) [Smith *et al.* (124)] and infection with the murin pneumonia virus [Weiss *et al.* (144)]. A similar effect in streptococcal infection in rats was observed by Reichlin & Glaser (104).

The effect of experimental hypothyroidism induced with thyrostatic drugs has also been studied in mice, and impairment has been noted in poliomyelitis by Holtman (36). Propylthiouracil has been credited with reducing in some measure the rate of paralysis in infection with Lancing poliomyelitis virus [Smith *et al.* (124)]. A favourable effect in infection with the murin pneumonia virus was observed by Weiss *et al.* (144) in mice after depression of the basal metabolism with radioactive iodine (I^{131}). Impairment in streptococcal infection in rats with experimental hypothyroidism was observed by Reichlin & Glaser (104).

Induced hyperthyroidism and hypothyroidism

In order to induce a state resembling hyperthyroidism in experimental animals, preparations of dried thyroid gland or purified preparations of thyroid hormone have been administered either by mouth or parenterally. The thyroid preparations have been given by mouth mixed with the food [Frugoni & Grixoni (30), Freud (29), Schröder (114), Schedtler (111, 112) and others], mixed in water to animals fed

by tube [Wasz-Höckert, Backman & Poppius (136) and others], or dissolved in the drinking water [Dubos (116), Nutter, Gemmill & Myrvik (90) and others]. Preparations were injected by Izzo & Cicardo (39), Swedberg (126), Lurie & Ninos (67) and others.

In the majority of investigations in which hyperthyroidism has been induced with various thyroid hormone preparations, only the purely »clinical» effect of the treatment has been studied. Symptoms such as weight loss, increasing restlessness, accelerated respiration, increased consumption of water and slight diarrhoea have been noted and the effect of the treatment has been evaluated on the basis of these criteria.

As a more accurate method for the evaluation of the effect of thyroid preparations on experimental animals, determination of the basal metabolic rate has been utilized. Krogh & Lindberg (55, 56) and Palmer & Leland (95) showed that in animals with an intact thyroid gland it is only possible to increase the basal metabolic rate to a certain degree by thyroid medication, an increase of 30—60 per cent being the maximum. These results have later been confirmed by Lurie & Ninos (67), among others. In his classical experiments of 1895, Magnus-Levy (79) obtained similar results when he gave large doses of thyroid to a normal subject and thus brought about an increase in oxygen consumption from 2.94 ml/kg to 3.50 ml/kg, which corresponds to an increase in basal metabolic rate of about 20 per cent.

In the same investigation Magnus-Levy (79) also showed that thyroid increases the basal metabolic rate by increasing the oxygen consumption. Subsequently the mechanism of the increased oxygen consumption observed in animals with experimental hyperthyroidism has been the subject of intensive research, but no satisfactory explanation has yet been advanced. Treatment with thyroid hormone cause a disturbance of the equilibrium between oxidative phosphorylation and oxidation in the cell by uncoupling of the former. It has been suggested that this change in the balance of the cell metabolism is responsible for the increased oxygen consumption [Martius & Hess (83, 84), Martius (82), Lardy (58), Rawson, Roll & Sonenberg (103), Klemperer (50)].

As previously mentioned, thyroidectomy has been utilized in order to induce hypothyroidism in experimental animals [Schedtler (111, 112), Izzo & Cicardo (39), Schröder (114), Lurie, Zapposodi & Blaker (69), Webb, Gilbert & Ryder (143), Kallós & Kentzler (41), Steinbach (125)]. Owing to their thyrostatic effect thiouracil preparations, both methyl- and propylthiouracil and thiourea derivatives, have also been

used for the same purpose [*e.g.* Meyer *et al.* (86), Eisman *et al.* (25), Lurie *et al.* (69), Schäfer (109), Wasz-Höckert *et al.* (136), Gädeke *et al.* (31)]. In thiouracil treatment the synthesis of thyroid hormone is inhibited, with the result that the basal metabolic rate is decreased [Lurie & Ninos (67) and others].

Metabolic rate and its determination

In his extensive survey of biological methods, Abderhalden (1, 2) analysed the principles underlying the different methods for determining basal metabolic rate in experimental animals. In direct calorimetry the heat given out by the animal during a given period is measured, in indirect calorimetry the amount of oxygen consumed and the quantity of carbon dioxide eliminated are determined and the metabolic rate is calculated from these values. An apparatus for the measurement of metabolic rate according to the principle of direct calorimetry was constructed by Tallqvist & Räihä (127). This apparatus is run electrically and works on the principle of differential calorimetry.

The basal metabolic rate in mice was determined by indirect calorimetry by Krebs (54), among others, who also utilized the results of Benedict (9). His results ranged between 145 and 148 Cal/kg/24 hrs., depending on the size of the animals. Kleiber (47, 48, 49), on the other hand, calculated basal metabolism as a function of weight and thus obtained values between 119 and 171 Cal/kg/24 hrs.

Experimental tuberculosis

The bacteriological methods used in the field of experimental research on tuberculosis have undergone considerable changes during the course of time and have been steadily improved. As mentioned in the foregoing, in the survey of studies concerning the influence of thyroid function on experimental tuberculosis, the determination of survival time and gross lesions has long been the dominant method. Feldman (26) and Karlson & Feldman (45) were the first investigators to utilize the evaluation of gross lesions as a criterion.

When the technique of intravenous inoculation had been adopted on a larger scale and rabbits and guinea-pigs had been replaced by mice as experimental animals, a method was elaborated in which the survival time was determined for large groups, standard inocula and their effect on the survival time being used as a control [Donovik (15), Rake *et al.*

(101), McKee *et al.* (78), Baker *et al.* (6), Youmans & Youmans (152, 153, 154), Batten & McCune (8), Zitrin & Wasz-Höckert (156)].

A method in which the development of gross lesions after intravenous inoculation of mice was used as a criterion was described by Youmans (150). Ceriotti (11) elaborated a method in which the changes in the weight of the lungs ensuing after intravenous inoculation were studied. A significant increase in weight correlated with the degree of severity of the tuberculous infection was reported.

Histopathological investigations of the lungs of intravenously inoculated mice have also been described [Youmans & Raleigh (151), Raleigh & Youmans (102)].

In 1949 Fenner, Martin & Pierce (27) described a method for the quantitative determination of the number of *M. tuberculosis* bacilli in both bacterial cultures and infected tissues. Since then Dubos and his team of the Rockefeller Institute and McDermot and his co-workers of Cornell University have elaborated and standardized a method for quantitative microbial determination of the extent of a tuberculous infection in animal tissues. This method has been employed in a large number of experiments by which the influence of different factors on the course of the infection has been studied [Dubos *et al.* (19, 22), Macaness *et al.* (80), Dubos *et al.* (20, 23), McCune *et al.* (76, 77), Wasz-Höckert *et al.* (140, 141) Jones *et al.* (40), Rhuland *et al.* (106), McCune *et al.* (73, 75), Wolinsky (145) and others].

Pierce, Dubos & Schaefer (98) published a detailed survey of the influence of different modes of inoculation on the course of infection. They concluded that the intravenous route yielded the most consistent results.

In investigations by Sever & Youmans (118, 119), the different phases of the quantitative microbial enumeration technique have been subjected to critical evaluation, and criteria for the statistical significance of the results have been suggested.

Kanai and Yaganisawa introduced a corresponding method for the quantitative determination of *M. tuberculosis* bacilli in organs of the guinea-pig and the rat [Kanai *et al.* (42, 43), Yaganisawa *et al.* (148, 149), Kanai *et al.* (44), Yaganisawa *et al.* (146, 147)].

After further elaboration of the method, Afflech *et al.* (3) demonstrated a good correlation between the count of *M. tuberculosis* bacilli visualized in the tissues by accurate staining methods and the results obtained with the enumeration technique.

Conge *et al.* (12) correlated different methods for the determination of experimental tuberculosis in mice, namely, the determination of survival time, the microbial enumeration technique and a histoquantitative method. A histoquantitative method of determination based on the line sampling technique [Uotila & Kannas (130)] has been elaborated by Kokkonen & Heikkilä (51).

The enumeration technique in non-tuberculous infections

The microbial enumeration technique has also been employed in the study of non-tuberculous infections. Staphylococcal infection and the factors influencing it have been subjected to investigation by Smith & Dubos (121, 122, 123), McCune *et al.* (74), Dubos & Schaedler (20, 21), Schaedler & Dubos (107, 108), Prigal & Dubos (100), Simon *et al.* (120), Wasz-Höckert *et al.* (137, 142, 138, 139), Hunt & Moses (38) and Kosunen (50), pneumococcal infection, streptococcal infection, dysentery and salmonella infection by Smith & Dubos (123), enterococcal and *Candida albicans* infection by Kosunen (52). The last mentioned writer also analysed the usefulness of the microbial enumeration technique in acute infections and simultaneously studied the errors inherent in the method in its different phases.

THE PRESENT STUDY

Object

The object of the present study was to investigate the effect of different degrees of induced hyperthyroidism and of hypothyroidism on the course of a chronic experimental *M. tuberculosis* infection in mice. This problem was approached

a) by inducing experimental hyperthyroidism of different degrees and hypothyroidism in mice and
b) by applying the microbial enumeration technique and the determination of survival time to the study of the course of a chronic infection with *M. tuberculosis* in mice, both in otherwise untreated animals and in animals with induced hyperthyroidism or hypothyroidism.

Material

Experimental animals

About 1 200 female mice belonging to a strain called Swiss Albino Webster, imported in 1956, were used in the experiments. On infection the animals weighed from 18 to 23 g, the mean weights of the animals employed in the different series of experiments ranging from 19 to 22 g.

The mice were housed in 5 litre glass jars with covers of wire netting, through which drinking water was supplied in pipette bottles. A maximum of 10—12 mice were kept in the same jar. The bottoms of the jars were covered with shavings, which were changed daily.

The temperature in the animal room was 19—21°C. The animals infected with *M. tuberculosis* were kept in a special cupboard fitted with glass doors and furnished with bactericidal ultraviolet lamps. No noxious effect of the ultraviolet light on the animals was observable.

The animals were fed on a standard pellet diet of constant composition *ad libitum* [Backman, Kosunen & Wasz-Höckert[1]) (5)]. Fresh drinking water was supplied daily in such quantities as the animals consumed.

Infective organism

A human strain of *M. tuberculosis* ($H_{37}Rv$) was used. It was imported in 1956 from the Cornell University Medical College, New York. It was maintained in oleic acid albumin [Dubos & Middlebrook (18)] by weekly passage to fresh medium, and incubated at 37°C.

Drugs

Thyroid powder (Orion)[2]). The brand employed contained 2.35 mg iodine per g = 0.23 per cent.

Methylthiouracil (Orion)[2]).

Methods

Bacterial cultures for inoculation

The *M. tuberculosis* strain ($H_{37}Rv$) was maintained in oleic acid albumin. A 1: 10 saline dilution of 7-day-old cultures was prepared and used as inoculum in those experiments in which the enumeration technique was employed. In the survival time experiments a stronger inocolum was used. This was prepared by homogenizing in saline with a teflon grinder [Pierce, Dubos & Schaefer (95)] colonies of the above-mentioned strain of *M. tuberculosis* which had been maintained on oleic acid albumin agar [Dubos & Middlebrook (18)].

Inoculation of mice

The mice were intravenously inoculated with 0.2 cc of one of the above-mentioned inocula in one of the lateral caudal veins. In order to facilitate intravenous injection the veins were dilated. In the first experiments the tails were dipped for one minute into water at 51°C, but this procedure was found occasionally to cause necrosis of the tail. Equally good dilatation of the caudal veins without any complications

[1] »Pellet diet», Orion Company, Pharmaceutical Manufacturers, Helsinki

[2] Orion Company, Pharmaceutical Manufacturers, Helsinki

was obtained when the animals were heated in their glass jars with an ordinary electric lamp for 15—20 minutes. This method has been described by Kosunen (52).

Administration of drugs

The drugs used in the experiments, thyroid and methylthiouracil, were given by mouth, homogeneously mixed by machine with the standard pellet diet on which the animals were fed.

Sacrifice of the animals

At the intervals required in the different experiments, 3—5 mice per experimental group were taken at random from different glass jars. They were killed with ether, fixed on cardboard disks with nails passed through the extremities and cleaned with ethyl alcohol. The skin was cut through with sterile instruments, and after renewed cleaning with alcohol the abdominal and thoracic cavities were opened aseptically and the spleen and lungs aseptically removed. Owing to the strictly aseptic technique employed, secondary infections were extremely rare. In those cases where secondary infection occurred, fungal infection was involved. With this technique, no transmission of infected material from one animal to another was possible.

Enumeration of the bacilli

In order to determine the volume of the organs removed, these were placed in graduated glass tubes containing 5 cc of sterile saline. When the volume had been read, the contents of the graduated glass tube, *i.e.* the organ and the saline, were poured into a sterile glass tube used in homogenization. The latter was carried out with the teflon grinder described and depicted in a paper by Pierce, Dubos & Schaefer (98). The rapidly rotating teflon rod pestle, which is run by an electric motor, was pressed up and down five times in the glass tube containing organ and saline, except in that experiment in which the effect of the grinding procedure on the number of culturable microbe units was studied. In this, the rod pestle was pressed up and down different numbers of times.

The enumeration of the bacilli was carried out by the plate counting method. The homogenates were serially diluted with saline, and three drops of 0.02 cc from each dilution were pipetted with a 0.2 cc micro-

pipette into Petri dishes with Dubos & Middlebrook's oleic acid albumin agar as culture medium. After 24 hours at 37°C the dishes were sealed with adhesive plaster, turned upside down and incubated at 37°C. After 21 days the colonies per drop were counted, and the result was calculated and expressed as the $\log_{10}$ of the culturable bacilli per ml of tissue.

Protective measures

Since virulent human *M. tuberculosis* bacilli ($H_{37}Rv$) were used, it was necessary to take effective steps to protect the personnel against infection. All work with infected material was carried out by persons wearing a protecting gown, a cap, a mask and rubber gloves. Furthermore, the work was performed in a transparent plastic hood, especially constructed for this purpose.

Technical errors inherent in the method

The microbial enumeration technique comprises a number of different phases. In order to form an opinion of the technical errors inherent in the method, all phases were analysed where this was possible.

Errors in the determination of the volume of the organs studied

The volume measurements were made twice by one and the same person; in the interval the order of the preparations was changed and the two readings were made independently of each other. On the basis of 82 such double determinations the following results were obtained, calculated according to the 95 per cent confidence interval method [Hald (34)]: 95 per cent confidence interval = $\pm$ 0.1 ml, standard deviation = 0.05 ml.

The effect of grinding

In order to find out how the grinding procedure influences the number of culturable *M. tuberculosis* bacilli or bacillary aggregates, an investigation was performed in which the preparations were ground 5, 10 and 20 times and samples of the respective products were cultured. A total of 38 determinations was made. The results were calculated by Student's t-test [Hald (34)]. No significant difference was demonstrable between the counts of *M. tuberculosis* bacilli per ml of tissue in samples taken after 5, 10 and 20 grindings. The t-value was 0.44 on comparison

of 5 and 10 grindings, 0.35 on comparison of 10 and 20 grindings and 0.07 on comparison of 5 and 20 grindings, which clearly shows that no significant differences existed.

Errors inherent in the plate counting method

The error inherent in the plate counting method employed is due in part to the error arising from pipetting of the dilutions and from the culture in Petri dishes, in part to the error resulting when the bacterial colonies are counted.

The error due to pipetting and culture was calculated on the basis of 50 double determinations of organ homogenates and determined by the 95 per cent confidence interval method [Hald (34)]: 95 per cent confidence interval = log unit — 0.3...+ 0.1, standard deviation = 0.098 log unit.

The error possibly resulting from the counting of the number of *M. tuberculosis* colonies in the Petri dishes was calculated on the basis of 57 double determinations made by one and the same person, the different readings being made independently of each other. The result calculated by the 95 per cent confidence interval method was: 95 per cent confidence interval = log unit — 0.039..+ 0.049, standard de viation = 0.022 log unit.

Summarizing, it may be stated that the error of the method for the different phases is as follows:

Volume determination: 95 per cent confidence interval $\pm$ 0.1 ml.

Effect of grinding: No significant difference between samples taken after 5, 10 and 20 grindings.

Culture and pipetting: 95 per cent confidence interval = log unit — 0.3... + 0.1.

Counting: 95 per cent confidence interval = log unit — 0.039... + 0.049.

It should be emphasized, however, that the variability of the biological factor, the experimental animal, constitutes the most significant component of the error of the method in experiments of this kind.

Induced hyperthyroidism and hypothyroidism

In order to evaluate the effect on the mice of the thyroid preparation (*Tyreoidin* Orion, containing 2.35 mg iodine per g = 0.23 per cent iodine) used in the experiments, its effect on untreated animals was studied.

Table 1. Amount of thyroid powder and methylthiouracil as a percentage of standard pellets and daily dosage per mouse.

	Thyroid powder % of standard pellets	Thyroid powder g/24 h.	Equal to iodine mg/24 h.
Control	—	—	0.044
Group 1	0.25 %	0.0087	0.054
» 2	0.5 »	0.017	0.089
» 3	1.0 »	0.035	0.14
» 4	2.0 »	0.07	0.23
» 5	4.0 »	0.14	0.37
» 6	— (Methylthiouracil 2 %)	—	0.044

On the basis of 10 days' experiments with 30 mice weighing 18—21 g it appeared that a mouse consumes an average of about 3.5 g standard pellets a day. The thyroid powder was homogeneously mixed in the standard pellet diet on which the animals were fed. The amounts of thyroid and iodine consumed are shown in Table 1.

From a chemical iodine analysis of the standard pellet diet with the addition of the various amounts of thyroid it was concluded that in 24 hours the animals received amounts of thyroid corresponding to the amount of iodine indicated in Table 1. These computations were done on the basis of analyses performed according to the usual pharmacological principles.[1])

During the last five years it has been found that the thyroid gland contains at least four metabolically active iodine thyronines, *i.e.* thyroxine or 3.5.3'5'-triiodothyronine, 3.5.3'- and 3.3'5'-triiodothyronine and 3.3'-diiodothyronine. Of these, the two latter are of little hormonal significance. However, 60—70 per cent of the total iodine content of the thyroid gland consists of metabolically inactive mono- and diiodothyrosine and a small amount (2—5 per cent) of iodine [Kyle, Canary, Meyer & Pac (57), Pitt-Rivers & Tata (99)]. Since the proportion of these components varies considerably in different preparations [Mandl & Block (81)], it was necessary to measure not only the iodine concentration, but also the biological effect on the experimental

[1] The analyses were performed by B. Anthoni, Ph.D., at the analytical laboratory of the Orion Company, Pharmaceutical Manufacturers, Helsinki

Table 2. Changes in metabolic rate corresponding to thyroid powder and methylthiouracil medication.

	Thyroid powder % of standard pellets	Changes in metabolic rate % of normal (Cal/kg/24h)		Mean
		Exper. I	Exper. II	
Control	—	(426 Cal/kg/24h)	(348 Cal/kg/24h)	387 Cal/kg/24h
Group 1	0.25 %	+11 %	+19 %	+15 %
» 2	0.5 »	+34 »	+48 »	+41 »
» 3	1.0 »	+44 »	+62 »	+53 »
» 4	2.0 »	+76 »	+98 »	+87 »
» 5	4.0 »	+88 »	+101 »	+94 »
» 6	— (Methylthiouracil 2 %)	—27 »	—13 »	—20 »

animals, of the preparations used. The determinations of the metabolic rate were performed with a differential calorimeter constructed by Tallqvist & Räihä (127). Owing to the construction of the apparatus the determinations were made on groups consisting of 10 animals. Measurement of the true basal metabolic rate was not attempted, since the animals had neither fasted prior to the experiments nor were immobile during them. Conditions being identical in all experiments, the results are fully comparable, however. Possible errors due to differences in the mobility of the animals, and errors which might result if the animals lay down on top of each other and thus failed to give out the normal amount of heat, were eliminated by placing all animals in isolated cells throughout the time of the experiment. At the time of determination the animals had been treated with thyroid or methylthiouracil for eight days. The results of calorimetry are indicated in Table 2, in which the means for the different determinations are given. In the control group the average metabolic rate was 387 Cal/kg/24 hrs.

From Table 2 it clearly appears that the thyroid medication which the animals received influenced their metabolic rate, increasing it to different degrees, depending on the amount of thyroid contained in their food. Dispersion in the individual experiments was rather wide, but the tendency, here represented by the mean of the different experiments, is obvious.

For the sake of comparison with the animals given thyroid, one group of animals was treated with melthylthiouracil (70 mg/24 hrs.). In calori-

metric experiments it was found that the metabolic rate of these animals fell by 20 per cent (Table 2).

The results of the calorimetric experiments here reported are also graphically depicted (Fig. 1).

The effect of thyroid medication on the *weight increase* of the animals was also studied. Simultaneously the »clinical» symptoms of hyperthyroidism developing during treatment were noted. These investigations were done on groups consisting of 30 mice. A few days after the institution of thyroid medication the first symptoms of hyperthyroidism were observable, namely, restlessness and increased consumption of water. After seven to eight days the full effect had been obtained. There were marked differences, however, between the different groups. It was found that the animals of group 5, which had received 4 per cent of thyroid in the food and whose metabolic rate had increased by 94 per cent, showed grave symptoms, *i.e.* an enormous thirst, some diarrhoea, greatly accelerated respiration and rather marked weight loss. After nine weeks' treatment all the 30 animals of this group had expired, and since no signs of infection were observable, thyrotoxicosis was concluded to be the probable cause of death. In group 4, in which the animals had received 2 per cent of thyroid and the metabolic rate was increased by 87 per cent, the symptoms were somewhat milder, but 10 animals out of 30 died during the 12 weeks of the experiment.

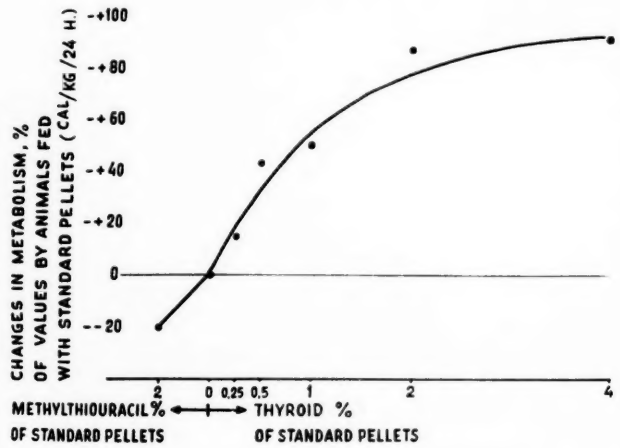

Fig. 1. Changes in metabolic rate in mice after thyroid powder and methylthiouracil medication.

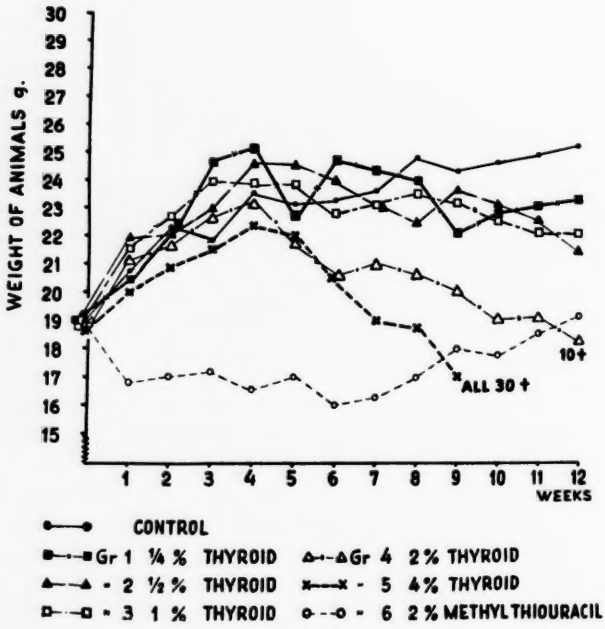

Fig. 2. Changes in weight of animals during thyroid powder and methylthiouracil medication.

In groups 2 and 3, given 0.5 and 1 per cent of thyroid, respectively, corresponding to an increase in metabolic rate of 41 and 53 per cent, the symptoms were milder still and the weight loss slight. No animals died from the treatment. The animals of group 1, which had received 0.25 per cent of thyroid, corresponding to an increase in metabolic rate of 15 per cent, were practically symptom-free, apart from a slightly increased consumption of water. In the group treated with methylthiouracil (2 per cent of the food), which resulted in a decrease in metabolic rate of 20 per cent, no appreciable symptoms were noted, but the animals lost some weight and remained definitely smaller than the controls.

Throughout the experimental period the animals were weighed once a week. The results calculated as means per group are depicted in Fig. 2.

It is seen that during the first few weeks of thyroid treatment the weight increase was almost normal, or perhaps in groups 1, 2 and 3 somewhat accelerated and in groups 4 and 5 somewhat retarded, whilst

the animals treated with methylthiouracil immediately began to lose weight. About four weeks after the institution of treatment the animals of groups 4 and 5 began to lose weight, and soon afterwards the first deaths occurred. About eight to nine weeks after the commencement of the experiment weight loss began in groups 1, 2 and 3, but no animals died.

Experimental tuberculosis

The evaluation of the course of a chronic tuberculous infection in mice was based on two main lines of study, namely, determination of the survival time for the strain ($H_{37}Rv$) of *M. tuberculosis* used, and determination of the number of bacilli per ml of tissue (spleen and liver) at different times after inoculation.

Survival time studies

For the purpose of determining the survival time for the strain of *M. tuberculosis* used in the experiments, $H_{37}Rv$ imported from Cornell University in 1956, the animals were intravenously inoculated with 0.2 ml of dilutions of cultures, containing 54.4×10^5 and 50.1×10^6 bacilli per ml. The time of observation was 12 weeks.

The results of the survival time investigations are depicted graphically in Fig. 3, in which the means of five experimental series, each

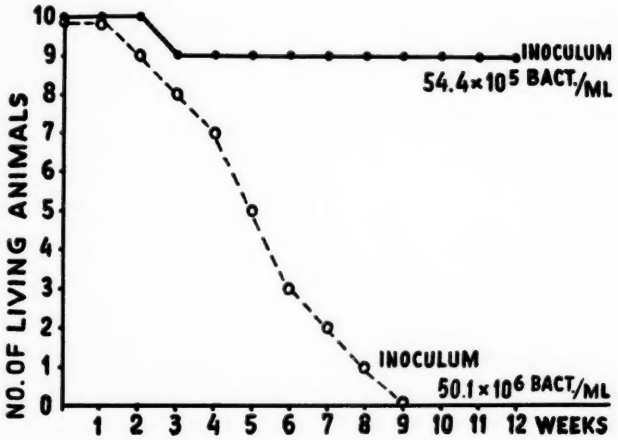

Fig. 3. Number of living animals at different times after challenge with 0.2 ml of suspensions containing 54.4×10^5 *M. tuberculosis* bacilli per ml and 50.1×10^6 *M. tuberculosis* bacilli per ml.

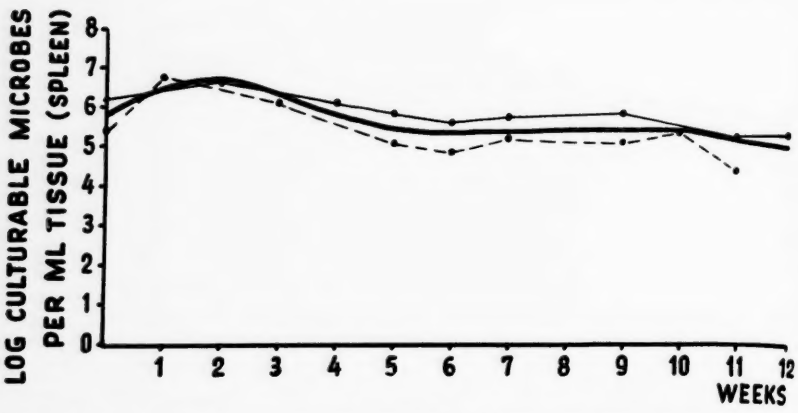

Fig. 4. Logarithms of the number of culturable *M. tuberculosis* bacilli per ml of spleen. Weekly means of two series are given and connected with fine lines. The trend line is heavier. Each symbol represents 5 animals.

comprising 10 animals per group, are indicated. It is seen that 0.2 ml of an inoculum containing 54.4×10^5 bacilli ($H_{37}Rv$) per ml caused a chronic, but not lethal, tuberculosis in the mice. An inoculum about ten times as strong, containing 50.1×10^6 bacilli ($H_{37}Rv$) per ml, caused a lethal infection.

Enumeration technique studies

In studying the course of a tuberculous infection in mice by the microbial enumeration technique the spleen and lungs were chosen for examination. During the 12-week period of the experiments, animals were taken at random at one- and two-week intervals, and the investigation was performed as described in the foregoing. The intravenous inoculum used was 0.2 ml of a *M. tuberculosis* suspension ($H_{37}Rv$) containing 26.2×10^5 bacilli per ml. The first determinations, which constitute the initial value, were made the day after inoculation. A total of 90 animals was used in these experiments.

Enumeration technique studies on the spleen

In Fig. 4 the course of infection in the spleen is graphically depicted; the time in weeks is plotted on the abscissa and the log of the number of culturable *M. tuberculosis* bacilli per ml of tissue on the ordinate. The mean values for the determinations per unit time in the two different series of this experiment have been plotted and each series of points

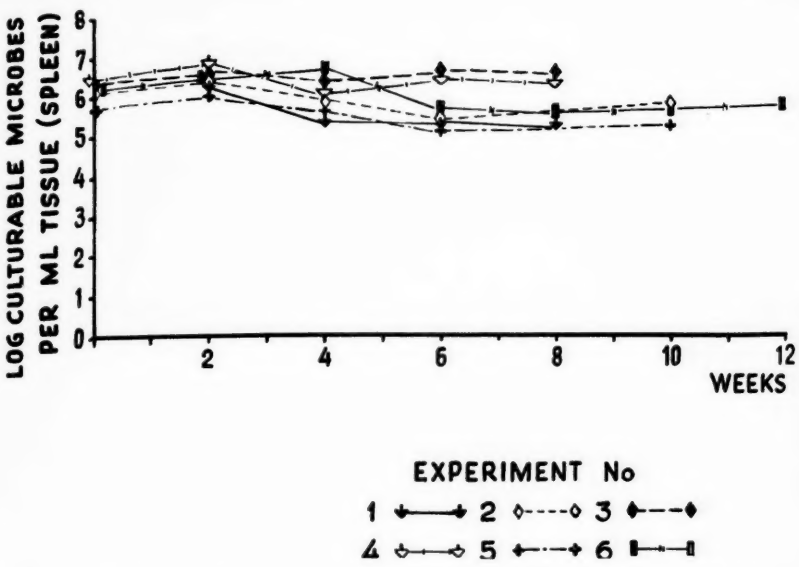

Fig. 5. Logarithms of the number of culturable *M. tuberculosis* bacilli per ml. of spleen. Weekly means of the control material in six experiments are given. Each symbol represents 3 animals.

connected with a separate thin line. A heavier »trend line», showing the general tendency in the experiments, has also been drawn.

It is seen from Fig. 4 that the number of culturable *M. tuberculosis* bacilli in the spleen remained largely the same throughout the whole of the 12-week experimental period, except for a slight increase during the first one or two weeks after inoculation.

In Fig. 5 a comparison is made between the control groups in the six experimental series in which the effect of thyroid medication was studied. The points represent the means of the logarithms of the numbers of culturable *M. tuberculosis* bacilli per ml of spleen tissue. The material comprised 93 inoculated, but otherwise untreated, animals.

It is seen from Fig. 5 that in these experiments the course of infection was practically the same as that shown in Fig. 4. On statistical analysis of the data the standard deviations for all determinations were calculated. The results, seen in Table 3, show that the six experiments in question are fully comparable with each other. For reasons to be discussed in what follows, this is very important from the standpoint of evaluation of the results.

Table 3. Standard deviation of the logarithms of the number of culturable *M. tuberculosis* per ml of spleen.

Time	Standard deviation
0 weeks	0.084 log unit
2	0.105
4	0.223
6	0.503
8	0.448
10	0.113
12	0.501

Enumeration technique studies on the lungs

On the same principles as described above, the course of infection in the lungs has been graphically depicted in Fig. 6.

Fig. 6 shows that in the lungs, as in the spleen, the number of culturable *M. tuberculosis* bacilli per ml of tissue remained fairly constant throughout the 12-week experimental period. It should be noted, however, that a steady, although slight, increase was observed for the first four or five weeks after inoculation, after which the count remained constant. Throughout the experimental period the total number of *M. tuberculosis* bacilli per ml of lung tissue was somewhat lower, however, than the corresponding value for the spleen. The material comprised 90 animals.

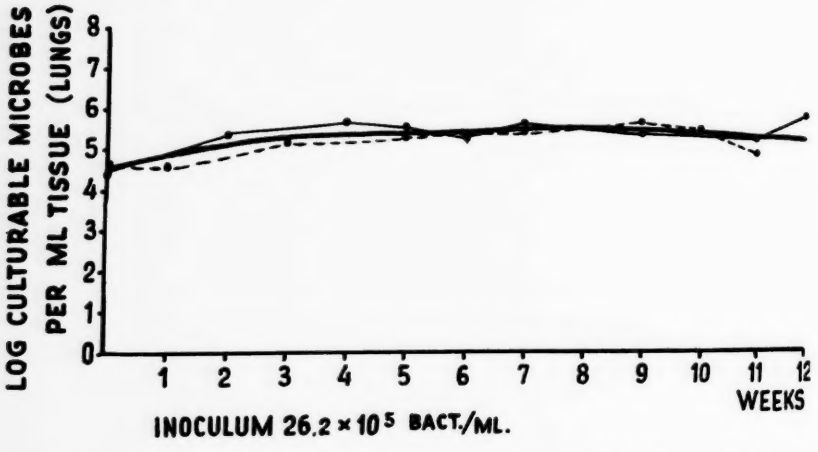

Fig. 6. Logarithms of the number of culturable *M. tuberculosis* bacilli per ml of lungs. See legend to Fig. 4.

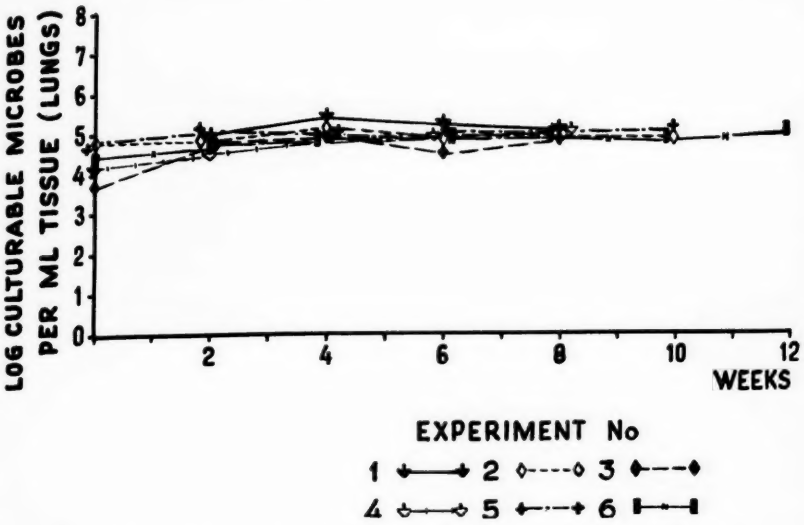

Fig. 7. Logarithms of the number of culturable *M. tuberculosis* bacilli per ml of lungs. See legend to Fig. 5.

Similarly, Fig. 7 shows graphically the logarithm of the number of culturable *M. tuberculosis* bacilli per ml of lung tissue in the inoculated, but otherwise untreated, control animals in those six experimental series in which the effect of thyroid medication on the course of the infection was studied. The material comprised 93 animals.

It appears from Fig. 7, in which the mean values for the observations per unit time in the various experiments are indicated, that the data for the normal material are practically identical in the six series. On statistical analysis of the data the standard deviations for all determinations were calculated. The results are seen in Table 4.

Table 4. Standard deviation of the logarithms of the number of culturable *M. tuberculosis* per ml of lungs.

Time	Standard deviation
0 weeks	0.209 log unit
2	0.279
4	0.204
6	0.181
8	0.044
10	0.299
12	0.422

When the spleens and lungs were removed, these organs were also examined macroscopically.

In the spleens, the first small visible tuberculous foci were observable some four to six weeks after inoculation. During the remainder of the experimental period the foci did not increase in number, and larger foci or coalescence of smaller ones was very seldom seen.

On gross inspection of the lungs no changes were observed.

Owing to the risk of error involved in the evaluation of gross lesions, in particular when the organs under study are as small as the present ones, the observations described above were not taken into account in the analysis of the results.

Effect of induced hyperthyroidism and hypothyroidism on experimental tuberculosis

Survival time studies

In order to study the effects of thyroid and methylthiouracil treatment on the survival time of mice infected with *M. tuberculosis* bacilli ($H_{37}Rv$), two series of experiments were made, one with an intravenous inoculum containing 54.4×10^5 bacteria per ml and another with an intravenous inoculum containing 50.1×10^6 bacteria per ml. Thyroid

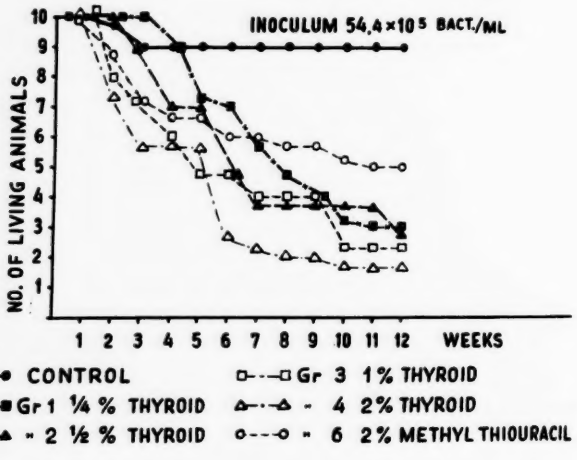

Fig. 8. Effect of thyroid and methylthiouracil medication on the number of living animals at different times after challenge with 0.2 ml of a suspension containing 54.4×10^5 *M. tuberculosis* bacilli per ml.

powder and methylthiouracil were given to the animals in the various groups of the different experimental series in the amounts indicated in Table 2, which also shows the biological effects of the thyroid and methylthiouracil medication. The material comprised 270 animals.

Fig. 8 is a graphical representation of the pooled results of the survival time experiments in which an inoculum containing 54.4×10^5 bacteria per ml was used.

During the experimental period of 12 weeks only a few of the animals in the control group (C) died, whereas the thyroid-treated animals showed a significantly higher mortality. No significant differences were observable between groups 1 (0.25 per cent of thyroid), 2 (0.5 per cent of thyroid), 3 (1 per cent of thyroid) and 4 (2 per cent of thyroid). By contrast, the death rate was significantly lower in group 6 (2 per cent of methylthiouracil). This group was included for the purpose of obtaining a preliminary idea of the extent to which mild hypothyroidism, in this case induced with methylthiouracil, influenced the course of infection.

The differences in survival time were tested by the χ^2 test [Hald (34)] at 12 weeks after inoculation. Between groups 1, 2, 3 and 4 no differences were observable, $\chi^2 = 1.55$ and n (the number of degrees of freedom) $= 3$. The difference between the control group and group 6 was highly significant, $\chi^2 = 11.43$ and $n = 1$. The difference between group 6 and groups 1, 2, 3 and 4 taken together was significant, $\chi^2 = 7.73$ and $n = 1$.

Fig. 9 shows graphically the mortality in those experiments in which an inoculum about ten times stronger was used, *i.e.* one containing 51.1×10^6 bacteria per ml.

It is clearly seen from Fig. 9 that the mortality was high in all groups. The control group C, group 4 (2 per cent of thyroid) and group 6 (2 per cent of methylthiouracil) form a uniform group inasmuch as no differences between them were observable when the results were calculated by the χ^2test, ($\chi^2 = 0.10$ and $n = 2$). Another group with uniform mortality was formed by groups 2 (0.5 per cent of thyroid), 3 (1 per cent of thyroid) and 5 (4 per cent of thyroid), between which no statistical differences were demonstrable, either. Between the control group and groups 4 and 6 taken together, and the control group and groups 2, 3 and 5 taken together, the difference was highly significant. ($\chi^2 = 12.44$ and $n = 1$). It is thus found that with an inoculum as strong as

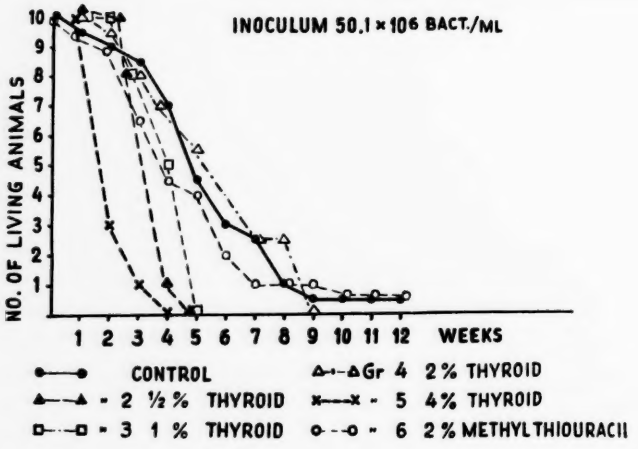

Fig. 9. Effect of thyroid and methylthiouracil medication on the number of living animals at different times after challenge with 0.2 ml of a suspension containing 50.1×10^6 *M. tuberculosis* bacilli per ml.

this, the animals die so soon that the effect of thyroid and methylthiouracil medication cannot be studied.

In summary, it may be stated on the basis of the survival time studies that thyroid medication, even in small doses causing only a slight increase in metabolic rate (group 1), caused an obvious aggravation of the course of a tuberculous infection in mice.

Enumeration technique studies

To study the course of a tuberculous infection in mice with experimental hyperthyroidism an inoculum ($H^{37}Rv$) was used, the potency of which was half that of the inoculum employed in the survival time studies, namely, 0.2 ml of bacterial culture dilution containing 26.2×10^5 bacteria per ml. In these experiments hyperthyroidism was induced by the following dosages of thyroid powder, mixed in standard pellets: in group 1, 0.25 per cent; in group 2, 0.5 per cent; in group 3, 1 per cent and in group 4, 2 per cent (Table 2).

Furthermore, one group (group 6) was treated with methylthiouracil in a pilot experiment in order to obtain an idea of the effect of hypothyroidism induced with methylthiouracil on the course of a tuberculous infection in mice as evaluated by the microbial enumeration technique (Table 2). The material comprised 260 animals.

Since with the technical resources available it was impossible to include all the above-mentioned six groups of animals in one and the same experiment, the study was carried out as a series of different experiments each comprising one infected, but otherwise untreated, control group (C) and two other groups. The inoculation dosage being constant, external conditions identical and the animal material homogeneous, it was found that in the control groups the course of the infection was practically identical in all experiments and could thus be reproduced (Tables 3 and 4, Figs. 5 and 7). Consequently, the results of the different experiments in the experimental series are comparable, and it is justifiable to draw conclusions from the pooled results.

The experiments lasted 8—12 weeks, and the number of culturable *M. tuberculosis* bacilli per ml of tissue was determined by the technique described in the foregoing. The spleen and lungs were chosen for investigation.

Investigation of the spleen

Fig. 10 is a graphical representation of the course of the infection in the spleen as evaluated by the microbial enumeration technique.

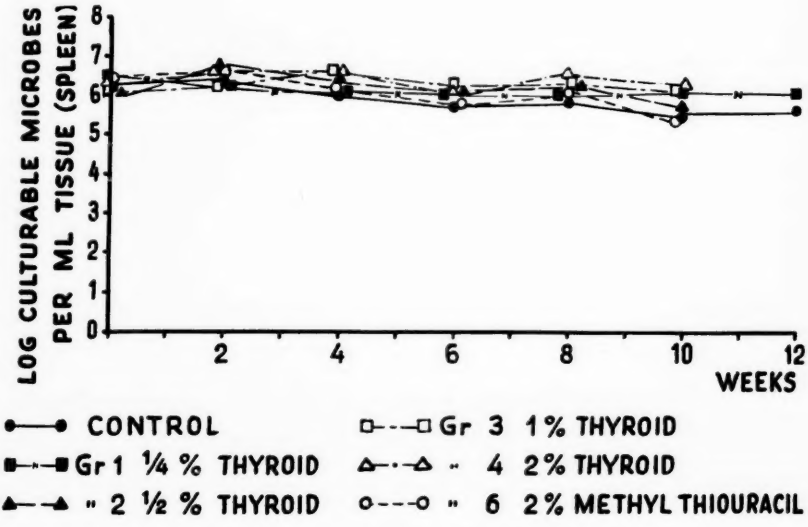

Fig. 10. Effect of thyroid and methylthiouracil medication on the logarithms of the number of culturable *M. tuberculosis* bacilli per ml of spleen. Weekly means are given. Each symbol represents 3 animals.

In Fig. 10 no differences between the different groups are revealed. The number of culturable *M. tuberculosis* bacilli thus seemed to be the same in the control group C and in the thyroid-treated groups 1, 2, 3 and 4 as well as in group 6, which was treated with methylthiouracil.

However, the results were also subjected to analysis of variance [Hald (34)] as shown in Table 5.

Table 5. Analysis of variance of the results of the enumeration technique studies on the spleen. All groups and times together.

Cause of variation	Degrees of freedom	Sum of squares	Mean square
Between groups	5	8.49	1.70
Between times	6	15.55	2.59
Interaction	24	4.47	0.19
Within groups and times	255	64.11	0.25
Total	290	92.62	

The variations between groups, F_1, and between times, F_2, were compared with the random variations. The corresponding variance ratios were

$$F_1 = \frac{1.70}{0.25} = 6.80 \quad \text{and} \quad F_2 = \frac{2.59}{0.25} = 10.36$$

The differences both between groups and between times are highly significant [Fisher & Yates (28)].

As a further measure, the analysis was repeated, the control group C and the time 0 being ignored. The result is shown in Table 6.

Table 6. Analysis of variance of the results of the enumeration technique studies on the spleen. All groups and times except for the control group (C) and the time 0.

Cause of variation	Degrees of freedom	Sum of squares	Mean square
Between groups	4	2.70	0.68
Between times	5	5.08	1.02
Interaction	14	1.18	0.08
Within groups and times	110	27.38	0.25
Total	133	36.34	

The variance ratios were now

$$F_1 = \frac{0.68}{0.25} = 2.72 \quad \text{and} \quad F_2 = \frac{1.02}{0.25} = 4.08$$

which shows that the differences between groups, F_1, were almost significant and between times, F_2, significant.

Finally the analysis was repeated without group 6. The result was now that shown in Table 7.

Table 7. Analysis of variance of the results of the enumeration technique studies on the spleen. All groups and times except for group C and group 6.

Cause of variation	Degrees of freedom	Sum of squares	Mean square
Between groups	3	1.22	0.41
Between times	5	3.01	0.60
Interaction	10	0.91	0.09
Within groups and times	91	24.73	0.27
Total	109	29.87	

The variance ratios were

$$F_1 = \frac{0.41}{0.27} = 1.52 \quad \text{and} \quad F_2 = \frac{0.60}{0.27} = 2.22$$

This means that no significant differences were observable between groups 1, 2, 3 and 4.

The difference between groups 1, 2, 3 and 4 and the control group was now tested. The result is seen in Table 8.

Table 8. Analysis of variance of the results of the enumeration technique studies on the spleen. Groups 1, 2, 3 and 4 taken together, and the control group (C).

Cause of variation	Degrees of freedom	Sum of squares	Mean square
Between groups	4	8.41	2.10
Between times	6	13.48	2.25
Interaction	19	4.19	0.22
Within groups and times	231	61.42	0.27
Total	260	87.50	

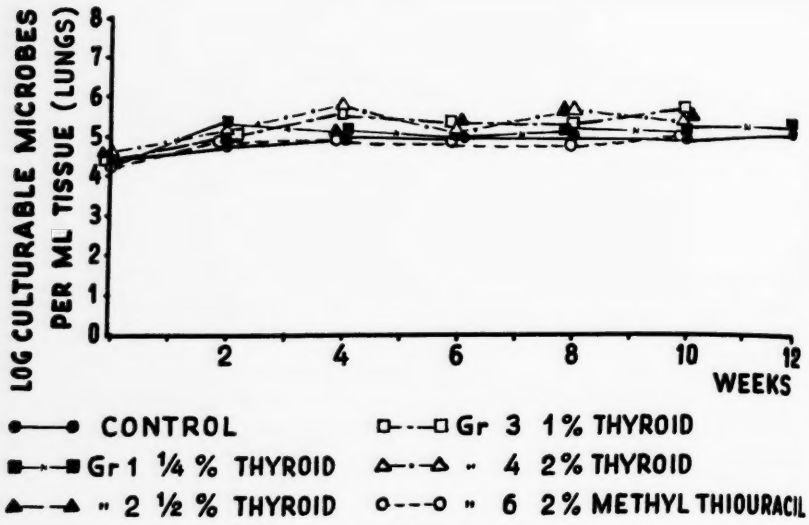

Fig. 11. Effect of thyroid and methylthiouracil medication on the logarithms of the number of culturable *M. tuberculosis* bacilli per ml of lungs. See legend to Fig. 10.

The variance ratios were

$$F_1 = \frac{2.10}{0.27} = 7.78 \quad \text{and} \quad F_2 = \frac{2.25}{0.27} = 8.33$$

The difference is highly significant.

Investigation of the lungs

Fig. 11 shows graphically the course of the infection in the lungs as revealed by the microbial enumeration technique.

The graphical representation in Fig. 11 reveals no differences between the groups. This seems to suggest that no differences exist in regard to the number of culturable *M. tuberculosis* bacilli between the control group C, the animals with induced hyperthyroidism (groups 1, 2, 3 and 4) and the animals with induced hypothyroidism (group 6).

However, the results appeared in a different light when an analysis of variance was performed as follows:

The variations between groups and between times were compared with the random variations as shown in Table 9.

Table 9. Analysis of variance of the results of the enumeration technique studies on the lungs. All groups and times together.

Cause of variation	Degrees of freedom	Sum of squares	Mean square
Between groups	5	6.49	1.30
Between times	6	21.66	3.61
Interaction	24	5.94	0.25
Within groups and times	245	41.70	0.17
Total	280	75,79	

The corresponding variance ratios were F^1 between groups and F^2 between times:

$$F_1 = \frac{1.30}{0.17} = 7.65 \quad \text{and} \quad F_2 = \frac{3.61}{0.17} = 21.24$$

The differences both between groups and between times are highly significant.

The analysis was repeated without control group C and the time 0. The result was then as shown in Table 10.

Table 10. Analysis of variance of the results of the enumeration technique studies on the lungs. All groups and times except for the control group (C) and the time 0.

Cause of variation	Degrees of freedom	Sum of squares	Mean square
Between groups	4	4.41	1.10
Between times	5	1.65	0.33
Interaction	14	2.99	0.21
Within groups and times	109	14.01	0.13
Total	132	23,06	

The variance ratios were now

$$F_1 = \frac{1.10}{0.13} = 8.46 \quad \text{and} \quad F_2 = \frac{0.33}{0.13} = 2.54$$

which shows that the differences between groups were highly significant and between times almost significant.

Finally the analysis was repeated without group 6. The result is shown in Table 11.

Table 11. Analysis of variance of the results of the enumeration technique studies on the lungs. All groups and times except for group C and group 6.

Cause of variation	Degrees of freedom	Sum of squares	Mean square
Between groups	3	0.26	0.09
Between times	5	1.85	0.37
Interaction	10	2.91	0.29
Within groups and times	92	9.32	0.10
Total	110	14.34	

The variance ratios were

$$F_1 = \frac{0.09}{0.10} = 0.90 \quad \text{and} \quad F_2 = \frac{0.37}{0.10} = 3.70$$

This means that no significant differences were observable between groups 1, 2, 3 and 4.

The difference between groups 1, 2, 3 and 4 and the control group is highly significant, as shown in Table 12.

Table 12. Analysis of variance of the results of the enumeration technique studies on the lungs. Groups 1, 2, 3 and 4 taken together, and the control group (C).

Cause of variation	Degrees of freedom	Sum of squares	Mean square
Between groups	4	4.43	1.11
Between times	6	21.05	3.51
Interaction	19	5.74	0.30
Within groups and times	224	35.56	0.16
Total	253	66.78	

The variance ratios were

$$F_1 = \frac{1.11}{0.16} = 6.93 \quad \text{and} \quad F_2 = \frac{3.51}{0.16} = 21.94$$

By statistical analysis of variance of the material it was thus found that in regard to both the spleen and the lung tissue there were statistically significant differences both between the various groups and between different points of time during the course of infection .The analysis showed that there were no statistical differences between the counts

of culturable *M. tuberculosis* bacilli from the spleen and lung tissue in the different groups with induced hyperthyroidism, groups 1, 2, 3 and 4. By contrast, the analysis of variance revealed that both in the control group (C) and in the animals with induced hypothyroidism (group 6) these organs exhibited a significantly lower number of culturable *M. tuberculosis* bacilli than they did in the animals with hyperthyroidism. Furthermore, it was found that in the animals with induced hypothyroidism the counts of culturable *M. tuberculosis* bacilli were significantly higher than in the control group C.

DISCUSSION AND CONCLUSIONS

The experimental hyperthyroidism induced by administration of various doses of thyroid powder caused measurable increases in the metabolic rate of the animals. In Table 2 it is seen that with increasing doses of thyroid the metabolic rate increased correspondingly, values up to + 92 per cent being noted. Investigations by Krogh & Lindberg (55, 56) and Palmer & Leeland (95) have shown that in euthyroid animals the maximum increase in basal metabolic rate obtainable by treatment with thyroid hormone is 60 per cent. The results noted in the present study are, perhaps, attributable to the fact that no basal metabolic rate proper was determined since the animals had neither fasted prior to the experiment, nor were immobile during the experiment. According to investigations by Benedikt (9), Krogh *et al.* (55, 56) and Kleiber (48, 49), mice have a basal metabolic rate of about 120—170 Cal/kg/24 hrs. For the above-mentioned reasons the metabolic rate of the normal material in the present study was 380 Cal/kg/24 hrs. In group 5 (Table 2) the mortality due to thyrotoxicosis was very high (Fig. 9). Hence this group was omitted from the experimental series in which the effect of induced hyperthyroidism on experimental tuberculosis was investigated.

The material also includes a group of animals (group 6 in Table 2) in which hypothyroidism was induced by methylthiouracil medication. This treatment caused a decrease in metabolic rate of 20 per cent. No data concerning measurements of metabolic rate in experimental animals made hypothyroid are available for comparison, however.

The microbial enumeration technique, which was employed as a bacteriological method, fully came up to the expectations raised by the relevant literature.The present investigation proved that with a standard inoculum ($H_{37}Rv$) and a homogeneous material of experimental animals (Swiss Albino Webster mice), the course of a chronic experimental

infection with *M. tuberculosis* in otherwise untreated animals is reproducible from one experiment to another (Tables 3 and 4, Figs. 5 and 7). Since the control groups thus exhibited an identical course of infection, it was possible to compare the results in the different series and also to compare groups which had not been included in the same experimental series. The error of the method was calculated for those phases of the investigation where this was possible, but the error due to individual variations between experimental animals which seem to be identical cannot be calculated.

From the results obtained the following conclusions can be drawn: Induced hyperthyroidism causing an increase in metabolic rate of 15—87 per cent aggravated the course of the infection, as compared with the control material. This is proved by the fact that the number of culturable *M. tuberculosis* bacilli per ml of spleen and lung tissue was significantly larger in the animals with hyperthyroidism than in the controls. No differences were demonstrable, however, between the various groups with different degrees of experimental hyperthyroidism. In animals with experimental hypothyroidism causing a decrease in metabolic rate of 20 per cent, the number of *M. tuberculosis* bacilli per ml of spleen and lung tissue was also significantly larger than in the controls, but significantly smaller than in the various groups of animals with induced hyperthyroidism.

The survival time experiments also showed that among the animals infected with *M. tuberculosis* the mortality was significantly higher in those treated with thyroid than in the untreated animals (Fig. 8). Furthermore, in animals made hypothyroid the survival time studies revealed a significantly higher mortality than in the control material of infected, but otherwise untreated, animals, but nevertheless a significantly lower mortality than that caused by the different degrees of hyperthyroidism.

These differences are statistically significant, although this is not clearly revealed by the graphs (Figs. 10 and 11). This appears to be due to the fact that graphs are based on arithmetical means, whilst an analysis of variance, which takes the dispersion more fully into account, allows of better evaluation of the results.

In summary, it may thus be stated that both induced hyperthyroidism and the degree of induced hypothyroidism investigated caused a significant increase in the number of *M. tuberculosis* bacilli per ml of lung and spleen tissue in mice as compared with the number observed

during the course of chronic experimental tuberculosis in otherwise untreated animals. The survival time studies showed that, in comparison with otherwise untreated animals, the mortality was significantly higher in the mice with various degrees of hyperthyroidism or with the degree of hypothyroidism investigated. In other words, both hyperthyroidism and hypothyroidism aggravated the course of an experimental infection with *M. tuberculosis* in mice. Similar results have been reported by Smith & Dubos (123) in studies on the effect of thyroid on a staphylococcal infection in mice.

As appeared from the survey of the literature, a large number of investigations on this subject have been published, but the results are conflicting. This seems to be due to the fact that both the technique of infection, the bacteriological methods used and the experimental animals have differed. The results of the present study showed that experimental conditions comparable with hyperthyroidism and hypothyroidism caused an increase in the number of *M. tuberculosis* bacilli per ml of tissue and an increased mortality among the experimental animals. In similar investigations concerning the relationship between the host organism and the infective agent it has been proved, however, that a variety of factors may influence the course of infection. Some of these factors have been identified already. Thus, it has been shown by Schaedler & Dubos (107) that fasting and a low-protein diet, by Wasz-Höckert *et al.* (138, 139, 142) that 5—8 per cent ethanol in the drinking water and zymosan injections, and by Prigal & Dubos (100) that allergic shock all exacerbate an experimental staphylococcal infection in mice. That heterologous vaccination causes aggravation of an experimental infection has been demonstrated in tuberculosis by Schröder (115), Gerniez-Rieux *et al.* (32), Parfentieff (96), Wasz-Höckert *et al.* (133, 134, 135) and Dubos & Schaedler (20). The last-mentioned investigators also showed that heterologous vaccines, endotoxin and bacterial constituents injected prior to inoculation of the experimental animals had a mitigating effect on the course of staphylococcal and tuberculous infections in mice. But if vaccination is performed simultaneously with or immediately after inoculation, experimental staphylococcal infection in mice is exacerbated. In guinea-pigs, Packalen (91) found that the course of experimental tuberculosis was aggravated by treatment with coli filtrations and staphylolysin, and Volkert *et al.* (131) reported a similar effect of an intercurrent viral infection in mice. The effect of a large number of different endocrinological factors on experi-

mental tuberculosis has been investigated. In regard to certain questions the evidence is conclusive. Thus, it has been established that adrenocortical hormone has a noxious effect in tuberculosis [Lurie (66) and others]. But there are other endocrinological factors, the influence of which in infection is not yet fully understood [Schäfer (110)]. In the relevant experiments the host organism and its immunological balance have been exposed to the influence of external agents, and the result of this »stress» has often been an aggravation of the course of the infection. Similarly in the present investigation, in which the host organism was exposed to endocrinological disturbance in the form of hyperthyroidism or hypothyroidism, the course of chronic, experimental tuberculosis in mice was exacerbated. One aspect has not been analysed, however, *i.e.*, the question of whether it was only the host organism, the mouse, that was influenced by the medication, or whether the virulence or other properties of the *M. tuberculosis* bacillus also changed. During the course of the study the colonies of *M. tuberculosis* were counted during different phases of the experiments, but no morphological differences between colonies from the normal material and colonies from animals made hyperthyroid or hypothyroid were observed.

The results of the present investigation show that induced hyperthyroidism and hypothyroidism led to aggravation of a chronic experimental infection with *M. tuberculosis* in mice, and that the number of culturable *M. tuberculosis* bacilli per ml of tissue increased in these conditions. This seems to indicate that the immunological balance or metabolism of the host organism was disturbed in such a way that the resistance to tuberculous infection was diminished. Similar results have previously been reported by Smith & Dubos (123) in investigations concerning the effect of induced hyperthyroidism on an experimental staphylococcal infection in mice, and by Wasz-Höckert (132) in studies on the effect of somatropic hormone on chronic experimental tuberculosis in mice. In these experiments the number of culturable *M. tuberculosis* bacilli per ml of tissue was not influenced, however, but survival time studies revealed an increased mortality among the hormone-treated animals.

SUMMARY

Experimental hyperthyroidism of several different degrees was induced in Swiss Albino Webster mice by giving the animals dried pulverized thyroid gland, and experimental hypothyroidism was induced with methylthiouracil. The thyroid and methylthiouracil preparations were homogeneously mixed with the standard pellets constituting the daily diet of the animals. The thyroid preparation was chemically analysed from the standpoint of iodine concentration, and its metabolic effect was determined by measurements of metabolic rate in a differential calorimeter for direct calorimetry. A total of five different degrees of experimental hyperthyroidism corresponding to increases in metabolic rate of 15 to 94 per cent were studied. The methylthiouracil medication induced hypothyroidism corresponding to a decrease in metabolic rate of 20 per cent.

Chronic experimental infection with *M. tuberculosis* ($H_{37}Rv$) was induced in the animals by intravenous inoculation with standard inocula. The course of the infection was studied in two organs, *i.e.* the spleen and the lungs, by the microbial enumeration technique. With the method employed the course of infection was reproducible from one experimental series to another during the period of 8—12 weeks that the experiments lasted.

The effect of different degrees of induced hyperthyroidism, corresponding to an increase in metabolic rate of 15 to 87 per cent, on chronic experimental infection with *M. tuberculosis* in mice was investigated by the microbial enumeration technique and survival time studies.

Furthermore, the effect of induced hypothyroidism, corresponding to a decrease in metabolic rate of 20 per cent, on the course of chronic experimental infection with *M. tuberculosis* in mice was studied by the same methods.

The results showed that both induced hyperthyroidism of different degrees of severity and the degree of induced hypothyroidism investigated clearly aggravated the course of a chronic experimental infection with *M. tuberculosis* in mice, *i.e.* there was an increase in mortality as evaluated by survival time studies. Investigations by the microbial enumeration technique showed that the number of culturable *M. tuberculosis* bacilli per ml of tissue (spleen and lungs) was larger in the animals with various degrees of induced hyperthyroidism or the degree of induced hypothyroidism investigated than in the controls.

It thus appeared that induced hyperthyroidism and hypothyroidism had a deleterious effect in experimental chronic infection with *M. tuberculosis* in mice. This may be interpreted as a sign that the immunebiological or metabolic balance of the host organism, the mouse, was disturbed by the hyperthyroidism or hypothyroidism with the result that the resistance to tuberculous infection was decreased.

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